VIROLOGY 235, 26–39 (1997)
ARTICLE NO. VY978668

Recombinant Human Immunodeficiency Pr55gag Virus-like Particles Presenting Chimeric

Ludwig Deml,* Reinhold Schirmbeck,† Jörg Reimann,† Hans Wolf,* and Ralf Wagner*,[1]

*Institute of Medical Microbiology, University of Regensburg, Franz-Josef-Strauss Allee 11, D-93053 Regensburg; and †Institute of Medical Microbiology and Immunology, University of Ulm, Albert-Einstein-Allee 11, D-89069 Ulm, Germany

Received March 7, 1997; returned to author for revision April 9, 1997; accepted June 9, 1997

Very recently, we demonstrated that the replacement of the human immunodeficiency virus type-1 (HIV-1) gp41 transmembrane protein by an Epstein–Barr virus gp220/350-derived membrane anchor resulted in the incorporation of chimeric envelope (Env) oligomers into Pr55^{gag} virus-like particles (VLPs), exceeding that of wild-type gp160 by a factor of 10. In this study, we examined the immunostimulatory properties of Pr55^{gag} VLPs to both (i) chimeric HIV-1 gp120 external envelope proteins and (ii) full-length gp160 presented on the outer surface of the particles. Immunization studies carried out with VLPs presenting different derivatives of the chimeric and wild-type Env proteins elicited a consistent anti-Pr55^{gag} as well as anti-Env antibody response in complete absence of additional adjuvants. In both cases, the immune sera exhibited an *in vitro* neutralizing activity against homologous HIV-1 infection in MT4 cells. Noteworthy, these VLPs were also capable of inducing a strong CD8$^+$ cytotoxic T-cell (CTL) response in immunized BALB/c mice that was directed toward a known CTL epitope in the third variable domain V3 of the gp120 external glycoprotein. However, the induction of V3-loop-specific CTLs critically depended on the amounts of Env proteins that were presented by the Pr55^{gag} VLPs. Moreover, the CD8$^+$ CTL response was not significantly altered by adsorbing the VLPs to alum or by repeated booster immunizations. These results illustrate that Pr55^{gag} VLPs provide a safe and effective means of enhancing neutralizing humoral responses to particle-entrapped gp120 proteins and are also capable of delivering these proteins to the MHC class I antigen processing and presentation pathway. Therefore, antigenically expanded Pr55^{gag} VLPs represent an attractive approach in the design of vaccines for which specific stimulation of neutralizing antibodies and cytotoxic effector functions to complex glycoproteins is desired.

INTRODUCTION

The type of immune response which leads to protection from an infection might be used as a guiding principle for the development of an efficient vaccine. In human immunodeficiency virus (HIV)-infected individuals, both humoral and cell-mediated immune responses are known to be induced, but the potential capacity of either component in preventing an HIV infection is poorly understood so far. Neutralizing antibodies appear to be an important component of a protective immune response, particularly in order to clear the virus or at least to reduce the viral load from the circulation (Ariyoshi *et al.,* 1992; Clementi *et al.,* 1993). Virtually all neutralizing activity in sera of HIV-1-infected humans is directed against the Env proteins gp120 and gp41 (Weiss *et al.,* 1985, 1986; Langlade Demoyen *et al.,* 1994). Neutralizing antibodies that were elicited after immunization of chimpanzees with HIV Env proteins protected these animals against an HIV infection (Berman *et al.,* 1990; Fultz *et al.,* 1992; Girard *et al.,* 1991; Mannhalter *et al.,* 1995). However, large-scale phase III field trials suggested only a limited — if any — effectiveness of recombinant gp120 subunit vaccines in humans (Macilwain, 1994). Potential disadvantages of this approach are the low complexity of these vaccine preparations and their inability to induce a strong cytolytic T-cell response.

The cell-mediated immunity has been demonstrated to play an essential role in limiting the severity and duration of a series of viral infections (Byrne and Oldstone, 1986; Quinnan *et al.,* 1982; Sethi *et al.,* 1983) and there is cumulative evidence to suggest that this may also be the case in HIV-infected individuals (Ariyoshi *et al.,* 1995; Borrow *et al.,* 1994; Plata *et al.,* 1987; Walker *et al.,* 1987). Gag-, Pol-, Env-, and Nef-specific cytolytic T-cells have been demonstrated early in the course of an HIV-1 infection, prior to the appearence of humoral response, and are presumed to control the initial viremia (Koup and Ho, 1994; Koup *et al.,* 1994; Safrit *et al.,* 1994). Studies on long-term nonprogressors (Cao *et al.,* 1995; Huang *et al.,* 1995), people at high risk (Langlade Demoyen *et al.,* 1994; Rowland-Jones *et al.,* 1995), uninfected children born of HIV-infected mothers (Bryson *et al.,* 1995; Cheynier *et al.,* 1992; De Maria *et al.,* 1994), and health care workers exposed to HIV-contaminated material (Pinto *et*

[1] To whom correspondence and reprint requests should be addressed at the Institute of Medical Microbiology, Klinikum Regensburg, Franz-Josef-Strauss Allee 11, 93053 Regensburg, Germany. Fax: ++49 (0) 941 944 6402. E-mail: Ralf.Wagner@rkmmhnw1.ngate.uni-regensburg.de.

0042-6822/97 $25.00

al., 1995) indicate that a $CD8^+$ cytotoxic T-lymphocyte (CTL) response is closely involved in delaying the onset of disease in HIV-infected subjects or even in the clearance of an initial HIV infection.

Therefore a promising HIV candidate vaccine should be capable of inducing both a strong cell-mediated immune response and high titers of neutralizing antibodies. Priming of cytolytic T-cells *in vivo* has been described for recombinant live viruses (Abimiku *et al.,* 1995; Cox *et al.,* 1993) and for proteins associated with detergent-type adjuvants such as liposomes (Lopes and Chain, 1992; Nair *et al.,* 1992; Reddy *et al.,* 1992), ISCOMs (Heeg *et al.,* 1991), or saponin QS-21 (Newman *et al.,* 1992). However, many of these approaches are, due to striking safety considerations, impractical for an application in humans.

Polyvalent particulate structures, mainly based on the hepatitis B (HB) virus surface antigen (Michel *et al.,* 1990; Schirmbeck *et al.,* 1994) and Ty virus-like particles (VLPs) (Griffiths *et al.,* 1991; Harris *et al.,* 1992), have been recently shown to elicit antibody, T-helper as well as cytotoxic T-cell responses. Reports suggesting that Gag-specific cellular immune responses may delay or abrogate disease progression (Cheingsong Popov *et al.,* 1991; Johnson *et al.,* 1991; Weber *et al.,* 1987) prompted others and us to develop an autologous, nonreplicating, and safe antigen delivery system, which is based on the particle-forming capacity of the HIV-1 $Pr55^{gag}$ precursor protein. Accordingly, recombinant $Pr55^{gag}$ VLPs have been used for the delivery of selected foreign epitopes (Griffiths *et al.,* 1993; Wagner *et al.*, 1993, 1994a,b) or complete polypeptides (Haffar *et al.,* 1991; Osterrieder *et al.,* 1995). The presentation of small epitopes derived from the HIV Env precursor gp160 by recombinant $Pr55^{gag}$ VLPs resulted in the induction of both humoral and cellular immune responses (Griffiths *et al.,* 1993; Wagner *et al.,* 1996). However, such particles elicited a strong antibody response against the $Pr55^{gag}$ polyprotein, while that directed to the inserted epitopes was weak (Luo *et al.,* 1992; Wagner *et al.,* 1996). Therefore, the incorporation of physiologically more relevant oligomeric HIV Env proteins may be advantageous for vaccine purposes, since they contain a variety of T-helper and CTL epitopes in addition to conformational epitopes known to be targets for cross-neutralizing antibodies (Sattentau and Moore, 1995; Fouts *et al.,* 1997). Very recently, we demonstrated that replacement of the gp41 transmembrane protein by an Epstein–Barr Virus gp220/350-derived membrane anchor resulted in the incorporation of chimeric envelope (Env) oligomers into $Pr55^{gag}$ VLPs in amounts significantly exceeding those of wild-type gp160 (Wagner *et al.,* 1995; Deml *et al.,* 1997). Herein, we describe the immunostimulatory properties of $Pr55^{gag}$ VLPs toward different variants of the HIV envelope proteins, which are stably anchored on the surface of VLPs. To our knowledge, this is the first report demonstrating that such particulate structures are — in the absence of adjuvants or replicating vector — capable of inducing a strong $CD8^+$ cytolytic T-cell response in addition to neutralizing antibodies toward glycoproteins that are exposed on the VLP surface. Thus, these genetically engineered, nonreplicating VLPs represent a promising candidate for the development of a safe and effective AIDS vaccine.

MATERIALS AND METHODS

Viruses and cells

$HIV\text{-}1_{LAI}$ was propagated and titrated on MT4 cells. MT4 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 medium containing 10% FCS. The $H\text{-}2^d$ B- lymphoma cell line A20 (TIB208) and the $H\text{-}2^d$ mastocytoma cell line P815 (TIB64) were also obtained from the ATCC. P815 and A20 cells were maintained at 37° in RPMI 1640 medium containing 5% FCS.

Monoclonal antibodies

The V3-specific murine monoclonal antibody (NEA 9305) recognizing a central motif of the V3-loop region (RIQRGPGRAFVTIGKI) was purchased from DuPont Canada, Inc. (Markham, Ontario). The p24(CA)-specific monoclonal antibodies (mabs) 13/5 and 16/4/2 have been previously mapped to amino acids 133–158 and 307–336 within the $Pr55^{gag}$ polyprotein (Wagner *et al.,* 1992).

Synthetic peptides and recombinant antigens

An 18-mer $V3_{LAI}$ peptide (SIRIQRGPGRAFVTIGKI) was synthesized in a 9050 peptide synthesizer and purified as described previously (Modrow *et al.,* 1989). Recombinant CHO cell-derived, purified $gp120_{LAI}$ was a gift of Genentech Inc. (San Franzisco, CA). Recombinant p24(CA) protein (pCO1) and heat inactivated HIV_{LAI} viruses were kindly provided by Matthias Niedrig (Behringwerke AG, Marburg, Germany).

Expression, purification, and biochemical characterization of hybrid $Pr55^{gag}$/Env VLPs

Baculoviruses (Ac-) expressing full-length $Pr55^{gag}$ (Wagner *et al.,* 1992) and gp160 as well as three different chimeric variants of the external HIV-1 glycoprotein gp120 were constructed as previously described in detail (Wagner *et al.,* 1995; Deml *et al.,* 1997). Briefly, chimeric glycoproteins consisted of the HIV external Env protein gp120 which was C-terminally covalently linked to the amino-terminus of the gp220/350 TM/CR moiety by a 6-amino acid (Ser-Gly-Ser-Gly-Ala-Gly) hinge region to allow independent folding of both domains (gp120-TM). Additional modifications include truncations of the gp120 carboxy-terminus by 5 amino acids (gp120-5TM) and by 20 amino acids (gp120-20TM) (Fig. 1). Hybrid $Pr55^{gag}$/Env VLPs were produced by coinfection of High Five insect cells with the recombinant baculoviruses AcPr55 and

Acgp120-TM, Acgp120-5TM, Acgp120-20TM, or Acgp160. Purification of VLPs was achieved by separating the concentrated cell culture supernatants on 10–60% sucrose gradients as previously described (Wagner *et al.,* 1994a). Hybrid $Pr55^{gag}$ VLP banded at the expected density of 1.13 to 1.19 g/cm^3, typical for HIV virions. The antigenic peak fractions were dialyzed against PBS. Using a Bio-Rad protein assay (Bio-Rad Laboratories, Germany) and a commercial p24 sandwich assay format (Abbott Laboratories, Abbott Park, IL), yields of VLPs were calculated to 2 to 3 mg/L cell culture supernatant. Analysis of the pooled antigenic peak fractions by SDS–10% PAGE followed by Coomassie staining detected comparable amounts of the unprocessed 55-kDa Gag precursor and demonstrated the VLP preparations to be >85% pure (Fig. 1B). Immunoblot analysis performed with a murine V3-loop-specific monoclonal antibody was required to visualize protein species corresponding to the molecular mass calculated for gp160 and the hybrid Env-TM derivatives in the antigenic peak fractions (Fig. 1C). Five- to tenfold higher yields of the chimeric glycoproteins (Env-TM; lanes 4–6) were regularly noted in the antigenic peak fractions when compared to wild-type Env proteins (lane 7). The concentration of the Env proteins incorporated into the VLP preparations was calculated by Western blot analysis and comparison with a calibration curve obtained after serial 2-fold dilutions (0.2–3.2 μg; lanes 8–12) of highly purified recombinant (r) gp120 protein (Genentech, Inc.). Additionally, a commercial gp120 capture ELISA kit (Intracel Corporation, Cambridge, MA) was used to verify the amounts of particle-entrapped Env proteins. Incorporation of Env-TM chimeras into the outer surface of VLPs was further confirmed by coimmunoprecipitation of $Pr55^{gag}$ and Env using gp120-specific monoclonal antibodies (data not shown) and by negative-staining immunogold electron microscopic analysis (Figs. 1D and 1E), which was performed essentially as described earlier (Osterrieder *et al.,* 1995). A more detailed immunoelectron microscopic analysis of VLPs presenting wild-type and chimeric gp120-TM proteins has been presented previously. Furthermore, chemical crosslinking analyses demonstrated that the Env-TM chimeras as well as the wild-type Env proteins are incorporated into VLPs as higher order oligomers (Deml *et al.,* 1997).

Immunization experiments

The feasibility of inducing an HIV-specific antibody response was analyzed by immunizing groups of three New Zealand White rabbits intramuscularly with 100-μg doses of recombinant antigens dissolved in 100 μl PBS. Following the priming immunization all animals received two boosts at Weeks 6 and 10 with the same dose of antigen. Induction of cytolytic T-cell responses was demonstrated in a BALB/c mouse model (Takahashi *et al.,* 1988). BALB/c mice ($H\text{-}2^d$) were bred under specific pathogen-free conditions. Twelve- to sixteen-week-old female BALB/c mice (3 to 5 mice per group) were injected with the indicated amounts of chimeric $Pr55^{gag}$/Env VLPs, $rgp120_{LAI}$, or a synthetic 18-mer $V3_{LAI}$ peptide (SIRIQRGPGRAFVTIGKI) in PBS. Adjuvants were only used where mentioned.

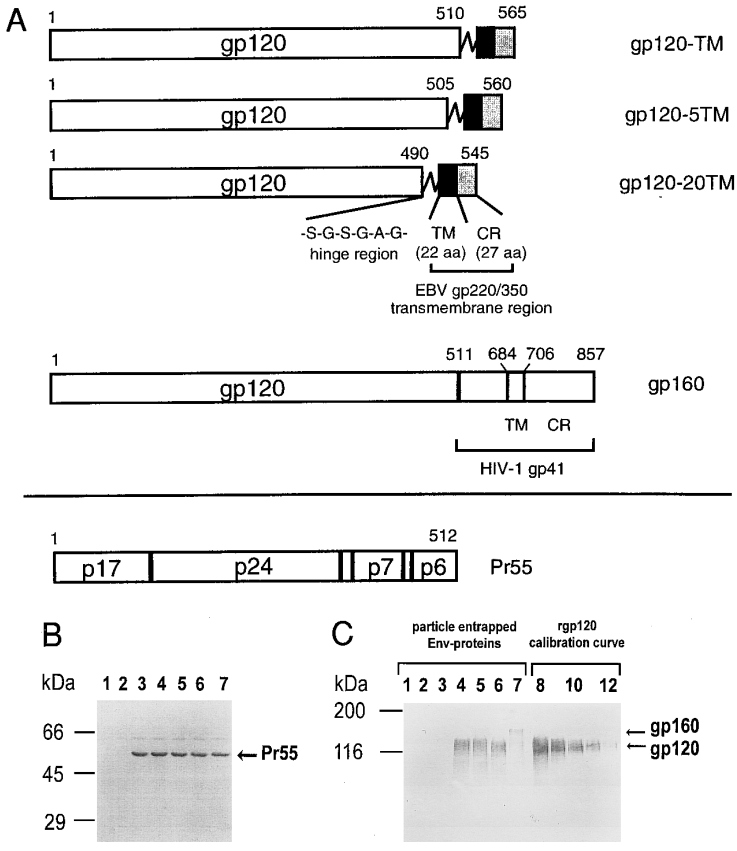
Enzyme-Linked Immunosorbent assay

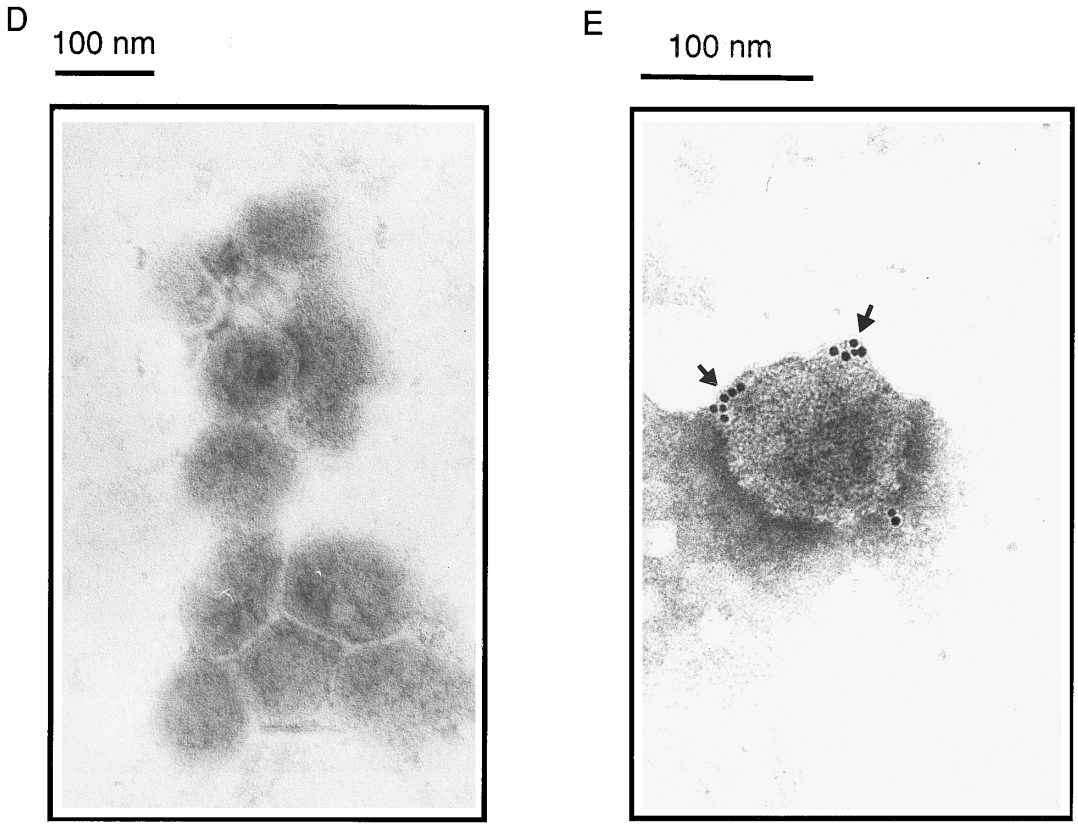
Rabbits were bled in 2-week intervals before and after the first immunization. Antibodies to inactivated $HIV\text{-}1_{LAI}$ virions, recombinant p24(CA), rgp120, or an 18-mer linear $V3_{LAI}$-loop-derived peptide were detected by enzyme-linked immunosorbent assay (ELISA) as previously described (Wagner *et al.,* 1996). Positive reactions were visualized with 0.1% hydrogen peroxide in the presence of 3′,3-diaminobenzidine tetrahydrochloride and stopped after 10 min with 1 *M* H_2SO_4. Absorbance was determined at 492 nm and reactions were considered positive if they exceeded the mean absorbance of equal dilutions of preimmune sera by a factor of 3.

HIV-1 neutralization assay

Antisera were heat inactivated at 56° for 30 min. Serial twofold dilutions of each serum were prepared in triplicate and incubated with 20 50% tissue culture infectious doses of $HIV\text{-}1_{LAI}$ for 1 hr at 37°. The virus–serum mixture was

FIG. 1. Hybrid $Pr55^{gag}$/Env virus-like particles used for immunization. (A) Schematic representation of wild-type HIV gp160 and chimeric Env-TM glycoproteins expressed on the outer surface of $Pr55^{gag}$ VLPs. The Epstein–Barr virus gp220/350-derived type I transmembrane domain (TM) and cytoplasmatic region (CR), as well as the amino acids (aa) of the flexible hinge region, are indicated. Abbreviations of the different chimeric genes are given at the right, the numbers of amino acids encoded by the chimeric genes are each indicated on the top of the constructs. The coexpression of these Env proteins and the particle-forming HIV $Pr55^{gag}$ polyprotein resulted in formation of $Pr55^{gag}$/Env chimeric VLPs. (B and C) Characterization of VLP preparations following sucrose density sedimentation. Recombinant $Pr55^{gag}$ VLPs (lane 3), VLPgp120-TM (lane 4), VLPgp120-5TM (lane 5), VLPgp120-20TM (lane 6), or VLPgp160 (lane 7) antigens were normalized for p24(CA) and fractionated by SDS–PAGE. Sedimented antigens of uninfected (lane 1) and wild-type baculovirus-infected High Five cells (lane 2) served as controls. Lanes 8–12 represent 3.2, 1.6, 0.8, 0.4, and 0.2 μg recombinant-made gp120, respectively. (B) Proteins were stained with Coomassie brilliant blue. (C) Alternatively, VLP-derived proteins as well as the serial twofold dilution of rgp120 were transferred onto a nitrocellulose filter and probed with mab NEA 9305 specific for the gp120 Env protein. Arrows at the right indicate the positions of the wild-type HIV Gag and Env proteins. The positions of the markers are given on the left (in kilodaltons). (D and E) Sucrose gradient sedimented $Pr55^{gag}$ VLP (D) and VLPgp120-TM (E) preparations were adsorbed to grids and incubated with anti-gp120-specific antibody. Bound mabs were detected with an anti-mouse IgG–gold conjugate and analyzed by negative-staining electron microscopy. (D) No specific surface labeling of particles was seen in negative controls. (E) VLP-entrapped immunogold-labeled gp120-TM proteins are indicated by black arrows.


A
1
510
565
gp120
gp120-TM
505
560
gp120-5TM
490
545
gp120-20TM
-S-G-S-G-A-G-
hinge region
TM
(22 aa)
CR
(27 aa)
EBV gp220/350
transmembrane region
511
684
706
857
gp160
TM
CR
HIV-1 gp41
512
p17
p24
p7
p6
Pr55
B
kDa
1 2 3 4 5 6 7
66
45
29
Pr55
C
particle entrapped
Env-proteins
rgp120
calibration curve
kDa
1 2 3 4 5 6 7 8 10 12
200
116
gp160
gp120


D
100 nm
E
100 nm

then added to 5×10^4 MT4 cells. Following an incubation at 37°, 5% CO_2 in a humid atmosphere for 6 days, cell cultures were examined for virus release by a commercial p24 sandwich assay (Abbott). The neutralizing titer was calculated as the reciprocal of the serum dilution that resulted in >90% reduction of the p24(CA) synthesis compared to the equivalent dilution of the preimmune serum from the same animal (Wagner *et al.,* 1996).

CTL assay

Single-cell suspensions were aseptically prepared from spleens of immunized mice 7 to 14 days postimmunization. Cells were suspended in α-MEM tissue culture medium (Gibco BRL, Eggenstein, Germany) supplemented with 10 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer, 5×10^{-5} β-mercaptoethanol, antibiotics, and 10% v/v FCS. Five percent (v/v) of a selected batch of Con A-stimulated rat spleen cell supernatants (Reimann *et al.,* 1988) was further added to the culture medium as a source of growth factors. Responder cells (3×10^7) were cocultured with 1.5×10^6 syngeneic, $V3_{LAI}$ peptide-pulsed P815 cells (irradiated with 20,000 rad) in 10 ml tissue culture medium in upright 25-cm^3 tissue culture flasks in a humidified atmosphere with 7% CO_2 at 37°. Cytotoxic effector populations were harvested after 6 days of *in vitro* culture and washed twice. Serial dilutions of effector cells were cultured with 2×10^3 target cells in 200-μl round-bottom wells. Targets were autologous A20 (H-2^d) cells (2×10^4/ml) incubated overnight at 37° with 10^{-8} *M* 18-mer $V3_{LAI}$ peptide. Non-peptide-pulsed cells were used as a negative control. Target and control A20 cells were labeled with ^{51}Cr (1 hr at 37°, 20 μCi/10^6 cells) and washed with cell culture medium prior to being added to the round-bottom wells. After a 4 hr incubation at 37°, 100 μl of supernatant was collected for gamma counting. The percentage specific release was calculated as [(experimental release − spontaneous release)/(total release − spontaneous release) × 100. Total counts were measured after adding 1% Triton X-100 to the labeled target cells. Spontaneously released counts were always less than 20% of the total counts. Data shown are the means of triplicate cultures. Standard errors of the means of triplicate data were always less than 20% of the mean.

RESULTS

Humoral immune responses of rabbits to hybrid $Pr55^{gag}$/Env VLPs

The capability of hybrid $Pr55^{gag}$/Env VLPs to induce $Pr55^{gag}$- and Env-specific antibodies was investigated in New Zealand White rabbits (Fig. 2). Six groups of three animals each received a priming immunization (im) of recombinant antigen (100 μg/dose) followed by two im boosts at Weeks 6 and 10 with the same dose. Control groups 1 and 2 were immunized by purified rgp120 or authentic $Pr55^{gag}$ VLPs without adjuvant. Groups 3–6 received different preparations of $Pr55^{gag}$/Env chimeric VLPs (VLPgp120-TM, VLPgp120-5TM, VLPgp120-20TM, and VLPgp160), also administered in absence of any adjuvant. Antibody titers to purified rp24(CA) and rgp120 were determined by ELISA. All groups of rabbits immunized with VLPs (groups 2–6) had already developed substantial levels of p24(CA)-specific antibodies 2 weeks after the first booster immunization (Fig. 2A). Additionally, high levels of gp120-specific antibodies were determined from sera derived from groups 3 to 6 (Fig. 2B). Reactive antibodies increased severalfold after the second booster immunization and reached $Pr55^{gag}$-specific endpoint titers of 1:16,000 to 1:64,000 (groups 2–6) and Env-specific titers of 1:16,000 to 1:64,000 (groups 3–6). Thus, despite the 5- to 10-fold varying concentrations of particle-entrapped Env proteins, VLPgp160 and VLPgp120-TM revealed a comparable capacity to induce gp120-specific antibodies. By contrast, Env-specific antibody responses raised in rabbits following repeated administration of rgp120 were low (group 1). Even after the second booster immunization the maximum titers of gp120-specific serum antibodies reached only 1:128.

The neutralizing properties of the rabbit antisera collected 2 weeks after the second booster immunization were assessed by measuring p24(CA) synthesis from HIV_{LAI}-infected MT4 cells. A murine gp120/V3-specific monoclonal antibody with a described neutralizing capacity of 1:200 against HIV-1_{LAI} (Ho *et al.,* 1987) was monitored in each experiment as a positive control. Titers were expressed as the serum dilution that reduced the release of p24(CA) from infected cells by >90% compared to the corresponding dilution of the preimmune serum. All $Pr55^{gag}$/Env-immunized rabbits (groups 3–6) developed neutralizing antibodies. The neutralizing capacity of the antisera correlated with the titers of gp120-specific antibodies but not with the antibody titers directed to the V3 loop within gp120 (Table 1). Rabbit sera derived from animals of groups 3 and 6 exhibited a substantial neutralizing capacity of 1:32–1:128 against infectivity of the homologous virus. A significant decrease in neutralization titers was monitored from antisera raised against VLPgp120-5TM and VLPgp120-20TM (groups 4 and 5). Antisera elicited to both $Pr55^{gag}$ VLPs and rgp120 (without adjuvant) did not block HIV_{LAI} infectivity (Table 1). $Pr55^{gag}$ VLPs are thus potent immunogens exhibiting intrinsic adjuvant properties for generating a humoral immune response with neutralizing activity to complex polypeptides (gp120-TM, gp160) that were presented on the surface of the VLPs.

Hybrid $Pr55^{gag}$/gp120-TM particles induce a specific MHC class I-restricted, $CD8^+$ CTL response in BALB/c mice

A H-2^d-restricted CTL epitope has been previously identified in the V3 loop of the HIV-1 exterior Env protein

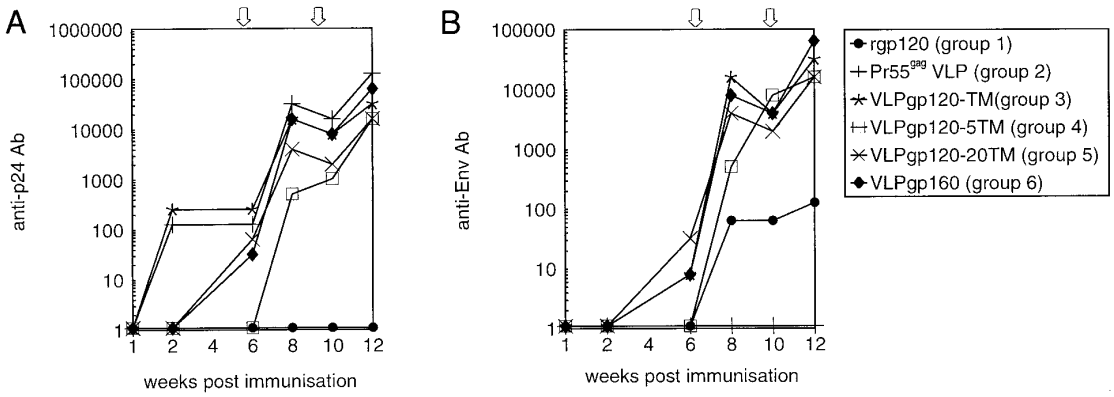


FIG. 2. Humoral immune response induced in rabbits following immunization with purified chimeric VLPs. Six groups of three rabbits each were immunized with 100 μg rgp120 (group 1), "naked" Pr55^{gag} VLPs (group 2), or various Pr55^{gag}/Env chimeric VLP preparations as indicated (groups 3–6). At 2-week intervals following the primary immunizations, serum samples were collected and assayed for HIV-1-specific antibodies by ELISA with (A) purified rp24(CA) or (B) purified rgp120. Endpoint antibody titers of the immune sera are depicted as the reciprocal of the last dilution giving the threefold optical OD compared to the preimmune sera. The arrows indicate the times of the secondary and tertiary immunizations. The mean endpoint titers of three rabbits in each group to the indicated antigens are shown.

gp120 (Takahashi *et al.,* 1988). In order to determine the capability of hybrid Pr55^{gag}/Env VLPs to induce a V3-specific CD8$^+$ CTL response, 20 μg of hybrid VLPgp120-TM was injected into BALB/c mice intraperitoneally (ip), subcutaneously (sc), or intravenously (iv). All immunizations were carried out in complete absence of adjuvant. Unprimed mice and mice immunized with 10 μg of HBsAg 22-nm subviral particles were used as negative controls. Seven days postimmunization spleen cells were obtained, transferred into culture, and restimulated with

TABLE 1

Serum Antibody and Neutralizing Antibody Titers of Rabbits Immunized with Hybrid Pr55^{gag}/Env VLPs

		Reciprocal maximum titers of serum antibodies[b]				
Rabbit group[a]	Antigen	HIV-1$_{LAI}$	P24(CA)	gp120	V3	NT[c]
1	rgp120	64	<16	128	<16	<8
		64	<16	128	<16	<8
		64	<16	64	<16	<8
2	Pr55^{gag} VLPs	32,000	32,000	<16	<16	<8
		64,000	32,000	<16	<16	<8
		32,000	32,000	<16	<16	<8
3	VLPgp120-TM	16,000	16,000	32,000	<16	128
		16,000	16,000	64,000	<16	128
		32,000	16,000	32,000	<16	32
4	VLPgp120-5TM	16,000	16,000	16,000	16	16
		8,000	8,000	16,000	32	16
		8,000	4,000	4,000	<16	<8
5	VLPgp120-20TM	64,000	32,000	16,000	128	32
		8,000	8,000	16,000	32	16
		16,000	16,000	16,000	16	16
6	VLPgp160	16,000	16,000	16,000	<16	32
		32,000	64,000	64,000	<16	128
		16,000	32,000	64,000	<16	32

[a] Five groups of three rabbits each were immunized intramuscularly at Weeks 0, 6, and 10 with 100-μg doses of different VLP preparations without adjuvant. All antigen preparations were diluted in PBS.

[b] Serum samples obtained 2 weeks after the second boost immunization were serially diluted and tested for HIV-1$_{LAI}$, p24(CA)-, gp120-, and V3-specific antibodies. ELISA titers are expressed as the reciprocal of the serum dilution that gave an absorbance value exceeding that obtained with the corresponding preimmune sera by a factor of 3.

[c] HIV-1 neutralizing assays were preformed with the homologous HIV-1$_{LAI}$ strain in MT4 cells. Neutralizing antibody titers (NT) were calculated by comparison with control wells of virus incubated with an adequate dilution of preimmuine sera and are expressed as the reciprocal of the serum dilution that inhibited p24(CA) synthesis by more than 90%. The commercial V3-specific monoclonal antibody NEA-9305 (DuPont) (Ho *et al.,* 1987) gave a reciprocal neutralization titer of 128 in this assay.

TABLE 2

PR55^{gag}/Env VLPs Efficiently Prime CD8$^+$ CTLs Irrespective of the Route of Immunization

Group[a]	Route of immunization	% Specific lysis[b] A20/Pep	% Specific lysis[b] A20
1	ip	52	2
2	sc	49	2
3	iv	40	3

[a] BALB/c mice were immunized with 20 μg of VLPgp120-TM antigens without adjuvants. Spleen cells obtained from mice 7 days postimmunization were restimulated *in vivo* with 18-mer V3-peptide-pulsed H-2^d P815 cells.

[b] The cytotoxic response was read out against H-2^d A20 cells pulsed for 1 hr with 10^{-8} *M* V3-loop-derived, synthetic 18-mer peptide (A20/peptide). The E:T ratio was 20:1.

syngeneic, peptide-pulsed, inactivated cells in a 5-day mixed lymphocyte-tumor cell culture (MLTC). The H-2^d mastocytoma cell line P815 pulsed with the synthetic 18-mer V3 peptide (SIRIQRGPGRAFVTIGKI) was used as stimulator cells in the MLTC. Cells harvested from MLTC were tested for specific cytotoxic activity in a standard short-term ^{51}Cr-release assay. Cells from mice primed by a single injection of 20 μg VLPgp120-TM developed specific CTL reactivity, irrespective of the route of antigen delivery. The *in vivo*-primed and *in vitro*-restimulated cells lysed peptide-pulsed but not unpulsed A20 targets. Identical results were obtained by using A20 cells, which were infected by gp120 recombinant vaccinia viruses, as targets in the chromium release assay (data not shown). As demonstrated earlier by others and by us, this confirmed that the 18-mer V3 peptides and Env recombinant vaccinia viruses were equally effective in promoting the MHC class I-restricted presentation of the antigenic peptide (Takahashi *et al.,* 1988; Wagner *et al.,* 1993; Griffiths *et al.,* 1993). Specific CTL reactivity was not detected in a primary MLTC in which spleen cells from unprimed BALB/c mice were cocultured with peptide-pulsed stimulator cells. *In vivo* priming of CTLs was thus required to induce the specific cytolytic response. Priming of mice with hybrid Pr55^{gag}/Env VLPs by the ip, sc, or iv route was equally efficient (Table 2). The CTL response was not enhanced by repeated booster injections. The effector cells expressed the CD3$^+$CD4$^-$CD8$^+$ phenotype, since they were eliminated by treatment with anti-CD8 antibody plus complement, but were not affected by treatment with anti-CD4 antibody plus complement (data not shown). In contrast, no V3-loop-specific cytolytic activity was observed in unprimed mice or in mice primed with HBsAg particles.

Low amounts of Env variants presented by VLPs prime V3-specific CTLs *in vivo*

To determine the minimum quantities of VLP-associated Env proteins necessary to prime a V3-specific CTL response *in vivo,* BALB/c mice were immunized ip with titrated doses of 100–1 μg of VLPgp120-TM and, for comparison, with the same doses of VLPgp160. The amounts of VLP-entrapped gp120-TM and gp160 were estimated on the basis of a gp120 calibration curve as described under Materials and Methods (Fig. 1C). Accordingly, the amounts of gp120-TM presented by the titrated doses of VLPs ranged from 5 μg gp120 presented by 100 μg VLPs down to 0.05 μg gp120 presented by 1 μg of hybrid VLPs (Table 3). By contrast, particles presenting gp160 were characterized by a 5- to 10-fold reduced incorporation into the VLPs. The amounts of gp160 presented by the titrated doses of VLPs ranged from 1 μg gp160 entrapped by 100 μg VLPs down to 0.01 μg gp160 presented by 1 μg of hybrid VLPs (Table 3). Clearly, the induction of a V3-specific CTL response correlated with the amount of presented Env proteins rather than with the total amount of the administered antigen. In both cases, CTL reactivity was reproducibly detected after a single low-dose injection of particles corresponding to about 0.2 μg of entrapped gp120-TM or full-length gp160. Lower doses of hybrid particles presenting less than 50 ng of Env-derived polypeptides were not sufficient to prime a V3-specific CTL response. No V3-specific CTL response was elicited in mice after administration with titrated doses of rgp120, not even with an excess of 25 μg of rgp120 per immunization (Table 3).

CTLs are primed by different variants of Pr55^{gag}/Env VLPs

To analyze the quality of the various Env constructs with respect to the induction of a primary V3-specific CTL response, the VLP preparations used for the immunizations were normalized to 1 μg of VLP-entrapped Env proteins as described under Materials and Methods (Fig. 1C). Accordingly, the total amount of antigens injected intraperitoneally into BALB/c mice was 100 μg for hybrid VLPgp160 and 20–25 μg for VLPs presenting the chimeric gp120 derivatives gp120-TM, gp120-5TM or gp120-20TM, respectively (Figs. 3E–3H). For controls, mice either were not immunized or were primed with a molar excess of 2.5 μg of a synthetic 18-mer V3 peptide (SIRIQRGPGRAFVTIGKI), 25 μg of purified rgp120, or 100 μg of Pr55^{gag} VLPs (Figs. 3A–3D). All antigen preparations were administered without any additional adjuvant. All tested Pr55^{gag}/Env VLP preparations elicited comparable levels of class I-restricted, V3-specific CTL reactivity. This indicated that the amount of particle-entrapped Env proteins rather than the total amount of administered VLPs determined the induction of V3-specific CTLs (Figs. 3E–3H). By contrast, no priming of V3-specific CTLs was detected even after immunization with a comparable dose of "naked" Pr55^{gag} VLPs or a high molar surplus of synthetic V3 peptide or rgp120 (Figs. 3B–3D).

TABLE 3

Low Doses of Pr55^{gag} VLP-Entrapped gp120-TM but Not Soluble rgp120 Proteins Prime a V3-specific CTL Response *in Vivo*

Group	Dose of VLPgp120-TM used for priming [a]	Corresponding quantities of particle-entrapped gp120-TM	% Specific lysis[b] A20/Pep	% Specific lysis[b] A20
1	100 μg	5 μg	52	2
2	20 μg	1 μg	45	2
3	4 μg	0.2 μg	18	3
4	1 μg	0.05 μg	5	2
Group	Dose of VLPgp160 used for priming[a]	Corresponding quantities of particle-entrapped gp160	% Specific lysis[b] A20/Pep	% Specific lysis[b] A20
5	100 μg	1 μg	50	2
6	20 μg	0.2 μg	16	4
7	4 μg	0.04 μg	4	3
8	1 μg	0.01 μg	5	2
Group		Dose of soluble rgp120 used for priming[a]	% Specific lysis[b] A20/Pep	% Specific lysis[b] A20
9		25 μg	7	2
10		5 μg	5	1
11		1 μg	6	3
12		0.2 μg	4	3
13		Nonimmunized	6	5

[a] Spleen cells of BALB/c mice primed by an ip injection of the dose of Pr55^{gag}/Env VLPs or rgp120 protein indicated. Spleen cells obtained from mice 14 days postimmunization were restimulated *in vitro* with 18-mer V3-peptide-pulsed H-2^d P815 cells.

[b] The cytotoxic response was read out against H-2^d A20 cells pulsed for 1 hr with 10^{-8} *M* V3-loop-derived, synthetic 18-mer peptide (A20/peptide). The E:T ratio was 20:1.

Primary and boosted responses of B-cells and CD8$^+$ CTLs of BALB/c mice to different antigen formulation

To compare the humoral (B-cell) and cytolytic T-cell response to different formulations of hybrid Pr55^{gag}/Env VLPs, mice were immunized intraperitoneally with 20 μg of VLPgp120-TM or 2.5 μg rgp120 in form of naked antigen, adsorbed to aluminum hydroxide (alum), or solubilized in SDS. A boost immunization with the same antigen formulation was given 3 weeks after the priming injection. The CTL reactivity to naked VLPgp120-TM was detectable as early as 6 days after the first immunization, persisted for many months, and was not enhanced by boost infections (data not shown). Three weeks after the booster immunizations, mice injected with hybrid VLPgp120-TM adsorbed to alum showed a CTL response which was comparable to that in animals primed with naked VLPs. For comparison, no V3-loop-specific CTLs were induced by injecting mice with rgp120 or rgp120 adsorbed to alum. Notably, vaccination with SDS-denatured hybrid particles or SDS-denatured rgp120 was at least equally effective in priming class I-restricted CTLs (Table 4). Hence, CTL priming by the native, particulate VLPgp120-TM and the SDS-solubilized particle component gp120-TM showed striking similarity. Surprisingly, in this system sensitization of the immune system with hybrid VLPs adsorbed to alum did not down-regulate the efficiency of priming a CD8$^+$ CTL response. These results contrast with previous observations, which indicate that alum or mineral oil significantly reduced the ability of chimeric VLPs, presenting the V3 epitope as part of a chimeric Pr55^{gag} polyprotein, to stimulate V3-specific CTLs *in vivo* (Griffiths *et al.,* 1993; Wagner *et al.,* 1996).

The B-cell response of BALB/c mice to VLPgp120-TM VLP formulations measured in terms of specific serum antibody levels appeared 1 to 3 weeks after the first immunization. Compared with the primary and boosted antibody responses of mice immunized with naked VLPgp120-TM, mice primed and boosted with hybrid particles adsorbed to alum showed significantly enhanced serum antibody levels, occuring even after the initial immunization. The maximum midpoint titers were 8- to 32-fold higher compared to the antibody levels detectable in mice immunized with hybrid VLPs alone. In contrast, the pretreatment of particles with SDS resulted in a significant reduction in their ability to stimulate a HIV-specific serum antibody response (Table 4).

DISCUSSION

Aiming at the development of more complex HIV candidate vaccines, we very recently suggested a novel strat-

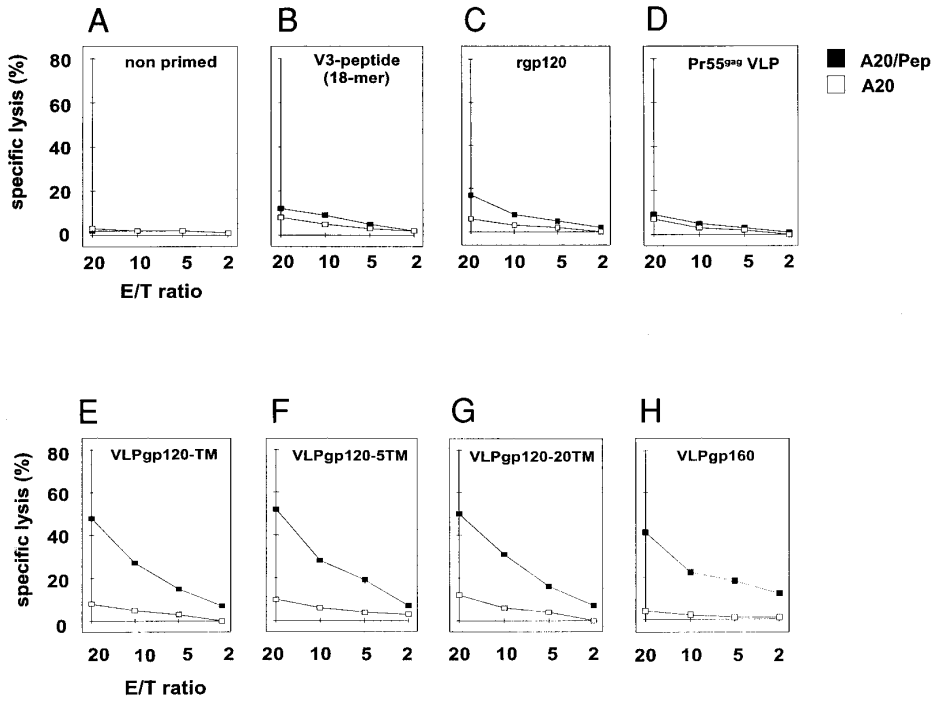


FIG. 3. Priming of class I-restricted cytotoxic T lymphocytes with different types of Pr55^{gag}/Env chimeric VLPs. BALB/c mice were immunized with an ip injection of (E–H) indicated Pr55^{gag}/Env VLP preparations each normalized to 1 μg of particle-associated Env proteins. Control mice were (A) not immunized or injected with (B) 10 μg of an 18-mer V3 peptide, (C) 25 μg rgp120, or (D) 100 μg naked Pr55^{gag} VLPs. Seven days pi spleen cells were obtained and specifically restimulated *in vitro* in a 5-day MLTC using syngeneic P815 mastocytoma cells pulsed with the synthetic V3 peptide as stimulator cells. The cytotoxic response was read against V3-peptide-pulsed (closed rectangles) or untreated (open rectangles) A20 H-2^{d} B-lymphoma cells as targets in a standard 4-hr ^{51}Cr-release assay. Data shown were mean values of triplicate cultures. The standard errors of the means of triplicate data were always less than 15% of the mean.

egy to incorporate high quantities of chimeric gp120 oligomers at the outer surface of highly immunogenic Pr55^{gag} virus-like particles (Deml *et al.,* 1997). Due to the absence of the authentic gp41 transmembrane protein, the VLP-associated chimeric gp120 polypeptides might possess some intrinsic advantages for vaccine purposes: (i) an exclusion of potential immunosuppressive effects and adverse immune reactions suggested to be induced by gp41 (Ruegg *et al.,* 1989; Ruegg and Strand, 1991; Yamada *et al.,* 1991), (ii) a 5- to 10-fold increased incorporation of oligomeric gp120 chimeras on the surface of immunostimulatory Pr55^{gag} VLPs compared to full-length gp160, and (iii) the absence of diagnostically relevant gp41-associated epitopes, which would allow one to distinguish vaccinees and HIV-infected people in future vaccination studies on a serological basis.

Herein, we describe the immunostimulatory properties of recombinant Pr55^{gag} VLPs to the HIV-1 external glycoprotein gp120 presented in large quantities on the outer surface of the particles. The magnitude of the antibody response to different preparations of purified Pr55^{gag}/Env VLPs was determined in New Zealand White rabbits. All groups of animals immunized with different types of Pr55^{gag}/Env particles developed substantial levels of gp120- in addition to Pr55^{gag}-specific antibodies immediately after the second immunization. These responses could be further increased following a third administration of hybrid VLPs. Additional boosts did not alter the antibody titers (data not shown). The presence of high titers of Env-specific antibodies was especially encouraging since the administered VLP preparations contained only low amounts of Env proteins (approximately 1–5 μg depending on the type of Pr55^{gag}/Env VLP). Moreover, administration of soluble rgp120 without adjuvant resulted in only a weak Env-specific antibody response, even following the second booster inoculation. These results are in agreement with previous studies, proposing that viral Env proteins presented in the context of particulate carriers or as aggregates enhance the stimulation of a specific immune response (Cabral *et al.,* 1978). Possibly, the polyvalent nature of the antigen facilitates an efficient uptake by antigen-presenting cells or is important for the cross-activation of different immune-competent cells.

Interestingly, only low titers of V3-specific antibodies were induced by immunizing rabbits with Pr55^{gag} VLPs presenting oligomeric Env proteins. This may be explained by previous reports demonstrating that the bulk of the V3 loop is masked on oligomeric forms of gp120. Consistent with this observation, immunization with oligomeric gp120 resulted in a low frequency of V3-loop reactivity, suggesting that the V3 loop may not be an

TABLE 4

Antibody and CTL Responses of BALB/c Mice to Different Formulations of Pr55^{gag}/gp120-TM VLPs

Group	VLPgp120-TM antigen formulation[a]	Serum antibody titers[b]			% Specific lysis[c]	
		HIV-1 lysate	p24(CA)	rgp120	A20/Pep	A20
1	Naked	512	1,024	1,024	46	1
2	Plus alum	16,000	16,000	8,200	43	6
3	Plus SDS[d]	128	128	64	55	3
Group	rgp120 antigen formulation[a]	Serum antibody titers[b]			% Specific lysis[c]	
		HIV-1 lysate	p24(CA)	rgp120	A20/Pep	A20
4	Naked	64	<16	64	3	1
5	Plus alum	1,028	<16	2,056	5	4
6	Plus SDS[d]	<32	<32	<32	59	3
7	Nonimmunized	<16	<16	<16	8	3

[a] BALB/c mice were immunized ip with 20 μg of VLPgp120-TM or 2.5 μg of rgp120 in form of naked antigen preparations (groups 1 and 4), adsorbed to alum (groups 2 and 5), or denatured in SDS (groups 3 and 6). A control group included unprimed BALBc mice (group 7). Three weeks after the priming immunization, mice received a boost injection with the same dose of antigen. Specific antibody and CTL levels were determined from immunized mice 3 weeks after the boost injection. The mean values of three individual mice in each group are shown.

[b] Serum samples obtained 3 weeks after the boost immunization were serially diluted and tested for HIV-$1_{LAI-,\ p24(CA)-}$, and gp120-specific antibodies by enzyme-linked immunosorbent assay. Titers are expressed as the reciprocal of the serum dilution that gave an absorbance value three times higher than the value obtained with the corresponding preimmune sera.

[c] Spleen cells obtained 3 weeks after the boost immunization were restimulated *in vitro* with 18-mer V3-peptide-pulsed H-2^d P815 cells. The cytotoxic response was read against H-2^d A20 cells pulsed for 1 hr with 10^{-8} *M* V3-loop-derived synthetic 18-mer peptide. The effector:target ratio was 20:1.

[d] Antigens were treated for 1 hr at 60° with 1% SDS and 2% mercaptoethanol as previously described (Schirmbeck *et al.,* 1995a).

immunodominant domain in oligomeric gp120 (Roben *et al.,* 1994, Sattentau and Moore, 1995; Fouts *et al.,* 1997). These observations are in agreement with our findings from chemical crosslinking experiments, indicating that gp160 as well as the Env-TM derivatives is presented on the VLPs in form of oligomeric (tetrameric) structures (Deml *et al.,* 1997).

The immunization of rabbits with Pr55^{gag}/Env chimeric particles also elicited antibodies that neutralized the infectivity of the homologous virus in MT4 cells. Sera taken from animals immunized with VLPgp160 revealed a virus neutralizing activity (<90%) at dilutions of up to 1:128, which was comparable to that reported in a previous study using VLPgp160 pseudovirions purified from a vaccinia virus-driven expression system as immunogens (Haffar *et al.,* 1991). However, identical neutralizing activities were also monitored in antisera obtained from rabbits after repeated administration of VLPs presenting the complete gp120 exterior domain on their surface (VLPgp120-TM). In both cases, the titers of neutralizing antibodies correlated rather with the titers of gp120-specific than with the titers of V3-loop-specific antibodies. By contrast antisera raised in animals after immunization with VLPs presenting gp120 derivatives that were truncated from the carboxy-terminus by 5 and 20 residues (gp120-5TM, gp120-20TM) exhibited only weak neutralizing activity. This is in agreement with observations by others, demonstrating that truncated Env glycoproteins induce — possibly as a consequence of improper folding — a humoral response that was characterized by low neutralizing activity although the titers of ELISA reactive antibodies were relatively high (Lu *et al.,* 1995). Therefore, in contrast to the truncated derivatives (gp120-5TM, gp120-20TM), incorporation of the complete HIV-1 exterior Env protein gp120 into Pr55^{gag} VLPs (VLPgp120-TM) by a heterologous transmembrane moiety resulted in the stimulation of substantial titers of neutralizing antibodies, even in the absence of the autologous gp41 transmembrane protein. Simultaneously, potential adverse side effects, suggested to be associated with parts of gp41 (Ruegg *et al.,* 1989; Ruegg and Strand, 1991; Yamada *et al.,* 1991), can be avoided.

Another novel aspect is that these lipoprotein particles can be used as a carrier component for the delivery of surface-fixed complex proteins to the MHC-I processing and presentation pathway. A low dose of Pr55^{gag}/Env particles injected into BALB/c mice without adjuvants efficiently primed HIV Env-specific CD8$^+$ CTLs. This response was detectable after a single ip, sc, or iv injection of 1 μg carrier-fixed Env proteins. Particle-entrapped chimeric gp120-TM derivatives and full-length gp160 were equally effective in priming of CD8$^+$ CTLs, when the doses of the immunogen were adjusted to the amount of particle-entrapped Env polypeptides (Fig. 3). However, in the case of identical doses of administered VLP protein, particles presenting the chimeric gp120-TM deriva-

tives were, obviously, due to an enhanced incorporation of the Env polypeptides (Fig. 1C), more efficient in inducing a V3-specific CTL response than was the VLPgp160 (Table 3). The CTL response was not inducible by injection of equimolar or even 20 times higher doses of V3-loop-derived peptides or rgp120.

The mechanisms underlying the *in vivo* priming of V3-specific $CD8^+$ CTL by hybrid $Pr55^{gag}$/Env VLPs are, however, still unclear at present. A trivial explanation for the *in vivo* priming of V3-specific $CD8^+$ CTL would be that a limited degradation of surface-exposed gp120 during the purification procedure or following immunization by extracellular proteases generates peptides that allow MHC class I-restricted presentation of the antigenic V3 peptide from an antigen-presenting cell (APC). In this case, one would expect to observe CTL priming by injecting equimolar amounts of the immunogenic peptide or rgp120. This was not observed in many independent experiments, even when a >100-fold higher dose of the rgp120 or of a synthetic 18-mer V3 peptide was used.

$Pr55^{gag}$ VLPs are lipoprotein spheres, and conjugation of proteins with lipids or liposomes has been previously demonstrated to facilitate their access to the endogenous processing pathway (Lopes and Chain, 1992; Martinon *et al.,* 1992; Schild *et al.,* 1991). The lipid components may therefore be supportive for the efficient uptake and/or entry of these antigens to the MHC class I processing pathway. However, particulate protein antigens, such as SDS or heat-denatured proteins (Bevan, 1987; Schirmbeck *et al.,* 1995a; Martinez-Kinader *et al.,* 1995), as well as Ty virus-like particles (Layton *et al.,* 1993), have also been demonstrated to be sufficient for the induction of $CD8^+$ CTLs. Thus, multimeric epitope presentation may also contribute to the efficiency of CTL priming by VLPs.

A VLP/gp160 consists of about 1500 $Pr55^{gag}$ precursor molecules and more than 200 copies of the Env protein as estimated by comparative immunoblotting of purified $Pr55^{gag}$/Env VLP's with HIV virions. Therefore a single uptake event introduces more than 200 copies of a CTL epitope into a single APC. As an estimated 50–100 MHC I molecules on the surface of an APC loaded with the relevant antigenic peptide are sufficient to trigger a CTL response, a single uptake event could potentially induce such a response. Whether or not the cytosol is the portal of this pathway is unclear at present. Alternatively, the hybrid $Pr55^{gag}$/Env VLPs may be processed in a novel endosomal/lysosomal processing pathway for class I-restricted presentation of peptides, as demonstrated for endocytosed 22 nm HBsAg particles (Schirmbeck *et al.,* 1995b).

The uptake of an exogenous viral protein into the endogenous pathway may be altered, when the protein is injected in conjunction with certain adjuvants. Previously we have demonstrated that immunization of BALB/c mice with different variants of $Pr55^{gag}$/V3 VLP adjuvanted with aluminum hydroxide or mineral oil significantly reduced or abrogated the induction of a specific $CD8^+$ CTL response (Wagner *et al.,* 1996). In contrast, no aluminum hydroxide-induced down-regulation of CTL priming was observed following an immunization with $Pr55^{gag}$/Env particles. These observations also reflect conflicting reports by others, demonstrating a suppressive or enhancing effect of adjuvants on the induction of cytotoxic T-cells, depending on the type of immunogen. Inefficient or absent CTL priming to antigen in alum or mineral oil has also been observed for Ty VLPs (Layton *et al.,* 1993) and HBsAg particles (Schirmbeck *et al.,* 1994), whereas poliovirus VP1 or Sendai virus-derived immunogenic peptides emulsified in complete Freund's adjuvants stimulated class I-restricted $CD8^+$ CTL response (Kast *et al.,* 1993; Kutubuddin *et al.,* 1992). An explanation would be that $Pr55^{gag}$/V3 VLPs, carrying a small V3 loop epitope within the particle, and hybrid $Pr55^{gag}$/Env VLPs, presenting the almost native gp120 or the complete wild-type gp160 on the surface of the particle, enter the APC by different portals. Additionally, different subsets of APC might be selected for the uptake and processing of these divergent types of VLPs.

In summary, this study demonstrates that HIV-1 $Pr55^{gag}$/Env chimeric VLPs administered in absence of any adjuvant are good immunogens with respect to both the induction of neutralizing antibodies and an HIV-specific CTL response against multiple epitopes. These results are in agreement with a similar strategy demonstrating that DNA immunization with Gag- and Env-expressing plasmids was also capable of stimulating both arms of the immune response (Lu *et al.,* 1995). Ongoing experiments using VLPgp120-TM preparations for immunogenicity studies in rhesus monkeys confirmed the induction of neutralizing antibodies and a CTL response to multiple epitopes in a primate model (manuscript in preparation) and thus recommend further evaluation of recombinant, chimeric VLPs as a potential HIV-1 candidate vaccine.

ACKNOWLEDGMENTS

The excellent technical assistance of Elke Perthen and Evelyn Kury is appreciated. We thank Genentech, Inc., for providing rgp120 and Dr. Susanne Modrow for delivering the synthetic peptides. This work was supported by the BMFT Grant 1 of the FVP BGA 1-88 and the DLR Grant 01 KI 9481/0 (AIDS Verbund Göttingen, Germany).

REFERENCES

Abimiku, A. G., Franchini, G., Aldrich, K., Myagkikh, M., Markham, P., Gard, E., Gallo, R. C., and Robert Guroff, M. (1995). Humoral and cellular immune responses in rhesus macaques infected with human immunodeficiency virus type 2. *AIDS Res. Hum. Retroviruses* 11, 383–393.

Ariyoshi, K., Harwood, E., Chiengsong Popov, R., and Weber, J. (1992). Is clearance of HIV-1 viraemia at seroconversion mediated by neutralising antibodies? *Lancet* 340, 1257–1258.

Ariyoshi, K., Cham, F., Berry, N., Jaffar, S., Sabally, S., Corrah, T., and

Whittle, H. (1995). HIV-2-specific cytotoxic T-lymphocyte activity is inversely related to proviral load. *AIDS* 9, 555–559.

Berman, P. W., Gregory, T. J., Riddle, L., Nakamura, G. R., Champe, M. A., Porter, J. P., Wurm, F. M., Hershberg, R. D., Cobb, E. K., and Eichberg, J. W. (1990). Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 345, 622–625.

Bevan, M. J.(1987). Antigen recognition. Class discrimination in the world of immunology. *Nature* 325, 192–194.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M., and Oldstone, M. B. (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* 68, 6103–6110.

Bryson, Y. J., Pang, S., Wei, L. S., Dickover, R., Diagne, A., and Chen, I. S. (1995). Clearance of HIV infection in a perinatally infected infant. *N. Engl. J. Med.* 332, 833–838.

Byrne, J. A., and Oldstone, M. B. (1986). Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus. VI. Migration and activity in vivo in acute and persistent infection. *J. Immunol.* 136, 698–704.

Cabral, G. A., Marciano Cabral, F., Funk, G. A., Sanchez, Y., Hollinger, F. B., Melnick, J. L., and Dreesman, G. R. (1978). Cellular and humoral immunity in guinea pigs to two major polypeptides derived from hepatitis B surface antigen. *J. Gen. Virol.* 38, 339–350.

Cao, Y., Qin, L., Zhang, L., Safrit, J., and Ho, D. D. (1995). Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* 332, 201–208.

Cheingsong Popov, R., Panagiotidi, C., Bowcock, S., Aronstam, A., Wadsworth, J., and Weber, J. (1991). Relation between humoral responses to HIV Gag and Env proteins at seroconversion and clinical outcome of HIV infection. *Br. Med. J.* 302, 23–26.

Cheynier, R., Langlade Demoyen, P., Marescot, M. R., Blanche, S., Blondin, G., Wain Hobson, S., Griscelli, C., Vilmer, E., and Plata, F. (1992). Cytotoxic T lymphocyte responses in the peripheral blood of children born to human immunodeficiency virus-1-infected mothers. *Eur. J. Immunol.* 22, 2211–2217.

Clementi, M., Bagnarelli, P., Menzo, S., Valenza, A., Manzin, A., and Varaldo, P. E. (1993). Clearance of HIV-1 viraemia after seroconversion. *Lancet* 341, 315–316.

Cox, W. I., Tartaglia, J., and Paoletti, E. (1993). Induction of cytotoxic T lymphocytes by recombinant canarypox (ALVAC) and attenuated vaccinia (NYVAC) viruses expressing the HIV-1 envelope glycoprotein. *Virology* 195, 845–850.

De Maria, A., Cirillo, C., and Moretta, L. (1994). Occurrence of human immunodeficiency virus type 1 (HIV-1)-specific cytolytic T cell activity in apparently uninfected children born to HIV-1-infected mothers. *J. Infect. Dis.* 170, 1296–1299.

Deml, L., Kratochwil, G., Osterrieder, N., Knüchel, R., Wolf, H., and Wagner, R. (1997). Increased incorporation of chimeric human immunodeficiency virus type I gp120 proteins into $Pr55^{gag}$ particles by an Epstein–Barr virus derived transmembrane domain. *Virology* 235, 10–25.

Fouts, T. R., Binley, J. M., Trkola, A., Robinson, J. E., and Moore, J. P. (1997). Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. *J. Virol.* 71, 2779–2785.

Fultz, P. N., Nara, P., Barre Sinoussi, F., Chaput, A., Greenberg, M. L., Muchmore, E., Kieny, M. P., and Girard, M. (1992). Vaccine protection of chimpanzees against challenge with HIV-1-infected peripheral blood mononuclear cells. *Science* 256, 1687–1690.

Girard, M., Kieny, M. P., Pinter, A., Barre Sinoussi, F., Nara, P., Kolbe, H., Kusumi, K., Chaput, A., Reinhart, T., Muchmore, E., *et al.* (1991). Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. *Proc. Natl. Acad. Sci. U. S. A.* 88, 542–546.

Griffiths, J. C., Berrie, E. L., Holdsworth, L. N., Moore, J. P., Harris, S. J., Senior, J. M., Kingsman, S. M., Kingsman, A. J., and Adams, S. E. (1991). Induction of high-titer neutralizing antibodies, using hybrid human immunodeficiency virus V3-Ty viruslike particles in a clinically relevant adjuvant. *J. Virol.* 65, 450–456.

Griffiths, J. C., Harris, S. J., Layton, G. T., Berrie, E. L., French, T. J., Burns, N. R., Adams, S. E., and Kingsman, A. J. (1993). Hybrid human immunodeficiency virus Gag particles as an antigen carrier system: Induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. *J. Virol.* 67, 3191–3198.

Haffar, O. K., Smithgall, M. D., Moran, P. A., Travis, B. M., Zarling, J. M., and Hu, S. L. (1991). HIV-specific humoral and cellular immunity in rabbits vaccinated with recombinant human immunodeficiency virus-like Gag–Env particles. *Virology* 183, 487–495.

Haffar, O. K., Moran, P. A., Smithgall, M. D., Diegel, M. L., Sridhar, P., Ledbetter, J. A., Zarling, J. M., and Hu, S. L. (1992). Inhibition of virus production in peripheral blood mononuclear cells from human immunodeficiency virus (HIV) type 1-seropositive donors by treatment with recombinant HIV-like particles. *J. Virol.* 66, 4279–4287.

Harris, S. J., Gearing, A. J., Layton, G. T., Adams, S. E., and Kingsman, A. J. (1992). Enhanced proliferative cellular responses to HIV-1 V3 peptide and gp120 following immunization with V3:Ty virus-like particles. *Immunology* 77, 315–321.

Heeg, K., Kuon, W., and Wagner, H. (1991). Vaccination of class I major histocompatibility complex (MHC)-restricted murine CD8+ cytotoxic T lymphocytes towards soluble antigens: Immunostimulating-ovalbumin complexes enter the class I MHC-restricted antigen pathway and allow sensitization against the immunodominant peptide. *Eur. J. Immunol.* 21, 1523–1527.

Ho, D. D., Sarngadharan, M. G., Hirsch, M. S., Schooley, R. T., Rota, T. R., Kennedy, R. C., Chanh, T. C., and Sato, V. L. (1987). Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. *J. Virol.* 61, 2024–2028.

Huang, Y., Zhang, L., and Ho, D. D. (1995). Characterization of nef sequences in long-term survivors of human immunodeficiency virus type 1 infection. *J. Virol.* 69, 93–100.

Johnson, R. P., Trocha, A., Yang, L., Mazzara, G. P., Panicali, D. L., Buchanan, T. M., and Walker, B. D. (1991). HIV-1 Gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes. Fine specificity of the Gag-specific response defined by using unstimulated peripheral blood mononuclear cells and cloned effector cells. *J. Immunol.* 147, 1512–1521.

Kast, W. M., Brandt, R. M., and Melief, C. J. (1993). Strict peptide length is not required for the induction of cytotoxic T lymphocyte-mediated antiviral protection by peptide vaccination. *Eur. J. Immunol.* 23, 1189–1192.

Koup, R. A., and Ho, D. D. (1994). Shutting down HIV. *Nature* 370, 416.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* 68, 4650–4655.

Kutubuddin, M., Simons, J., and Chow, M. (1992). Poliovirus-specific major histocompatibility complex class I-restricted cytolytic T-cell epitopes in mice localize to neutralizing antigenic regions. *J. Virol.* 66, 5967–5974.

Langlade Demoyen, P., Ngo Giang Huong, N., Ferchal, F., and Oksenhendler, E. (1994). Human immunodeficiency virus (HIV) nef-specific cytotoxic T lymphocytes in noninfected heterosexual contact of HIV-infected patients. *J. Clin. Invest.* 93, 1293–1297.

Layton, G. T., Harris, S. J., Gearing, A. J., Hill Perkins, M., Cole, J. S., Griffiths, J. C., Burns, N. R., Kingsman, A. J., and Adams, S. E. (1993). Induction of HIV-specific cytotoxic T lymphocytes in vivo with hybrid HIV-1 V3:Ty-virus-like particles. *J. Immunol.* 151, 1097–1107.

Lopes, L. M., and Chain, B. M. (1992). Liposome-mediated delivery stim-

ulates a class I-restricted cytotoxic T cell response to soluble antigen. *Eur. J. Immunol.* 22, 287–290.

Lu, S., Santoro, J. C., Fuller, D. H., Haynes, J. R., and Robinson, H. L. (1995). Use of DNAs expressing HIV-1 env and noninfectious HIV-1 particles to raise antibody responses in mice. *Virology* 209, 147–154.

Luo, L., Li, Y., Cannon, P. M., Kim, S., and Kang, C. Y. (1992). Chimeric Gag–V3 virus-like particles of human immunodeficiency virus induce virus-neutralizing antibodies. *Proc. Natl. Acad. Sci. USA* 89, 10527–10531.

Macilwain, C. (1994). US puts large-scale AIDS vaccine trials on ice as 'premature.' *Nature* 369, 593.

Mannhalter, J. W., Fischer, M. B., Wolf, H. M., Kupcu, Z., Barrett, N., Dorner, F., Eder, G., and Eibl, M. M. (1995). Immunization of chimpanzees with recombinant gp160, but not infection with human immunodeficiency virus type 1, induces envelope-specific Th1 memory cells. *J. Infect. Dis.* 171, 437–440.

Martinez-Kinader, B., Lipford, G. B., Wagner, H., and Heeg, K. (1995). Sensitization of MHC class I-restricted T cells to exogenous proteins: Evidence for an alternative class I-restricted antigen presentation pathway. *Immunology* 86, 287–295.

Martinon, F., Gras Masse, H., Boutillon, C., Chirat, F., Deprez, B., Guillet, J. G., Gomard, E., Tartar, A., and Levy, J. P. (1992). Immunization of mice with lipopeptides bypasses the prerequisite for adjuvant. Immune response of BALB/c mice to human immunodeficiency virus envelope glycoprotein. *J. Immunol.* 149, 3416–3422.

Michel, M. L., Mancini, M., Riviere, Y., Dormont, D., and Tiollais, P. (1990). T- and B-lymphocyte responses to human immunodeficiency virus (HIV) type 1 in macaques immunized with hybrid HIV/hepatitis B surface antigen particles. *J. Virol.* 64, 2452–2455.

Modrow, S., Hoflacher, B., Mertz, R., and Wolf, H. (1989). Carrier-bound synthetic peptides. Use as antigen in HIV-1 ELISA tests and in antiserum production. *J. Immunol. Methods* 118, 1–7.

Nair, S., Zhou, X., Huang, L., and Rouse, B. T. (1992). Class I restricted CTL recognition of a soluble protein delivered by liposomes containing lipophilic polylysines. *J. Immunol. Methods* 152, 237–243.

Newman, M. J., Wu, J. Y., Gardner, B. H., Munroe, K. J., Leombruno, D., Recchia, J., Kensil, C. R., and Coughlin, R. T. (1992). Saponin adjuvant induction of ovalbumin-specific CD8+ cytotoxic T lymphocyte responses. *J. Immunol.* 148, 2357–2362.

Osterrieder, N., Wagner, R., Brandmuller, C., Schmidt, P., Wolf, H., and Kaaden, O. R. (1995). Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp14 (gB). *Virology* 208, 500–510.

Pinto, L. A., Sullivan, J., Berzofsky, J. A., Clerici, M., Kessler, H. A., Landay, A. L., and Shearer, G. M. (1995). Env-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. *J. Clin. Invest.* 96, 867–876.

Plata, F., Autran, B., Martins, L. P., Wain Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J. M., and Debre, P. (1987). AIDS virus-specific cytotoxic T lymphocytes in lung disorders. *Nature* 328, 348–351.

Quinnan, G. V.,Jr., Kirmani, N., Rook, A. H., Manischewitz, J. F., Jackson, L., Moreschi, G., Santos, G. W., Saral, R., and Burns, W. H. (1982). Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N. Engl. J. Med.* 307, 7–13.

Reddy, R., Zhou, F., Nair, S., Huang, L., and Rouse, B. T. (1992). In vivo cytotoxic T lymphocyte induction with soluble proteins administered in liposomes. *J. Immunol.* 148, 1585–1589.

Reimann, J., Bellan, A., and Conradt, P. (1988). Development of autoreactive L3T4+ T cells from double-negative (L3T4−/Ly−2-) Thy-1+ spleen cells of normal mice. *Eur. J. Immunol.* 18, 989–999.

Roben, P., Moore, J. P., Thali, M., Sodroski, J., Barbas, C. F. I., and Burton, D. R. (1994). Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. *J. Virol.* 68, 4821–4828.

Rowland-Jones, S., Sutton, J., Arijoshi, K., Dong, T., Gotch, F., Mo-Adam, S., Whitby, D., Sabally, S., Allimore, A., Corrah, T., Taciguchi, M., Schultz, T., McMichal, A., and Whittle, H. (1995). HIV-specific cytotoxic T-cells in HIV exposed but uninfected Gambian woman. *Nature Med.* 1, 59–64.

Ruegg, C. L., Monell, C. R., and Strand, M. (1989). Inhibition of lymphoproliferation by a synthetic peptide with sequence identity to gp41 of human immunodeficiency virus type 1. *J. Virol.* 63, 3257–3260.

Ruegg, C. L., and Strand, M. (1991). A synthetic peptide with sequence identity to the transmembrane protein gp41 of HIV-1 inhibits distinct lymphocyte activation pathways dependent on protein kinase C and intracellular calcium influx. *Cell Immunol.* 137, 1–13.

Safrit, J. T., Andrews, C. A., Zhu, T., Ho, D. D., and Koup, R. A. (1994). Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: Recognition of autologous virus sequences within a conserved immunodominant epitope. *J. Exp. Med.* 179, 463–472.

Sattentau, Q. J., and Moore, J. P. (1995). Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. *J. Exp. Med.* 182, 185–196.

Schild, H., Norda, M., Deres, K., Falk, K., Rotzschke, O., Wiesmuller, K. H., Jung, G., and Rammensee, H. G. (1991). Fine specificity of cytotoxic T lymphocytes primed in vivo either with virus or synthetic lipopeptide vaccine or primed in vitro with peptide. *J. Exp. Med.* 174, 1665–1668.

Schirmbeck, R., Deml, L., Melber, K., Wolf, H., Wagner, R., and Reimann, J. (1995a). Priming of class I-restricted cytotoxic T lymphocytes by vaccination with recombinant protein antigens. *Vaccine* 13, 857–865.

Schirmbeck, R., Melber, K., Mertens, T., and Reimann, J. (1994). Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: Implication for the pathogenesis of HBV-induced hepatitis. *J. Virol.* 68, 1418–1425.

Schirmbeck, R., Melber, K., and Reimann, J. (1995b). Hepatitis B virus small surface antigen particles are processed in a novel endosomal pathway for major histocompatibility complex class I-restricted epitope presentation. *Eur. J. Immunol.* 25, 1063–1070.

Sethi, K. K., Omata, Y., and Schneweis, K. E. (1983). Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus-specific and H-2-restricted cytotoxic T lymphocytes. *J. Gen. Virol.* 64, 443–447.

Takahashi, H., Cohen, J., Hosmalin, A., Cease, K. B., Houghten, R., Cornette, J. L., DeLisi, C., Moss, B., Germain, R. N., and Berzofsky, J. A. (1988). An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 85, 3105–3109.

Wagner, R., Fliessbach, H., Wanner, G., Motz, M., Niedrig, M., Deby, G., von Brunn, A., and Wolf, H. (1992). Studies on processing, particle formation, and immunogenicity of the HIV-1 *gag* gene product: A possible component of a HIV vaccine. *Arch. Virol.* 127, 117–137.

Wagner, R., Boltz, T., Deml, L., Modrow, S., and Wolf, H. (1993). Induction of cytolytic T lymphocytes directed towards the V3 loop of the human immunodeficiency virus type 1 external glycoprotein gp120 by $p55^{gag}$/V3 chimeric vaccinia viruses. *J. Gen. Virol.* 74, 1261–1269.

Wagner, R., Deml, L., Fliessbach, H., Wanner, G., and Wolf, H. (1994a). Assembly and extracellular release of chimeric HIV-1 $Pr55^{gag}$ retrovirus-like particles. *Virology* 200, 162–175.

Wagner, R., Deml, L., and Wolf, H. (1994b). Polyvalent, recombinant HIV-1 virus-like particles: Novel HIV-1 vaccine strategies. *Antibiot. Chemother.* 46, 48–61.

Wagner, R., Deml, L., Fitzon, T., and Wolf, H. (1995). "Vaccines 95: Molecular Approaches to the Control of Infectious Diseases" (R. M. Chanock, F. Brown, H. S. Ginsberg, and E. Norrby, Eds.), pp. 347–356. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wagner, R., Deml, L., Schirmbeck, R., Niedrig, M., Reimann, J., and Wolf, H. (1996). Construction, expression and immunogenicity of chimeric HIV-1 virus-like particles. *Virology* 220, 128–140.

Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S., and Schooley, R. T. (1987). HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* 328, 345–348.

Weber, J. N., Clapham, P. R., Weiss, R. A., Parker, D., Roberts, C., Duncan, J., Weller, I., Carne, C., Tedder, R. S., Pinching, A. J., *et al.,* (1987). Human immunodeficiency virus infection in two cohorts of homosexual men: Neutralising sera and association of anti-Gag antibody with prognosis. *Lancet* 1, 119–122.

Weiss, R. A., Clapham, P. R., Cheingsong Popov, R., Dalgleish, A. G., Carne, C. A., Weller, I. V., and Tedder, R. S. (1985). Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients. *Nature* 316, 69–72.

Weiss, R. A., Clapham, P. R., Weber, J. N., Dalgleish, A. G., Lasky, L. A., and Berman, P. W. (1986). Variable and conserved neutralization antigens of human immunodeficiency virus. *Nature* 324, 572–575.

Wolf, H., Modrow, S., and Wagner, R. (1994). "Retroviruses of Human AIDS and Related Animal Diseases" (R. Odile, Ed.), pp. 299–307. Colloque Des Cent Gardes, Marnes-La-Coquette, Paris.

Yamada, M., Zurbriggen, A., Oldstone, M. B., and Fujinami, R. S. (1991). Common immunologic determinant between human immunodeficiency virus type 1 gp41 and astrocytes. *J. Virol.* 65, 1370–1376.